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Original Article

Identification of Main *Brucella* species Implicated in Ovine and Caprine Abortion Cases by Molecular and Classical Methods

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Abstract

Brucellosis is recognized as a major public health concern leading to critical economic losses in livestock animals. The present study assessed *Brucella* spp. isolated from aborted ovine and caprine fetuses in different parts of Iran between 2016 and 2019. It used classic and molecular methods in order to determine the *Brucella* species carrying higher risks of abortion complications in these animals. A total of 189 samples from 35 cases/case series from milk (16 sheep, and 8 goats), 19 abomasum content (sheep), and 146 aborted fetuses (116 sheep, and 30 goats) were bacteriologically examined. Subsequently, the resultant *Brucella* isolates were further characterized by phenotypic and molecular approaches. The multiplex Polymerase chain reaction (PCR) (Bruce-ladder) and IS711-based PCR were performed on all the extracted DNA to evaluate the presence of *Brucella* spp. As suggested by the obtained results, all recovered isolates from ovine and caprine abortion samples were either *B. melitensis or B. abortus*. An issue of concern was the implication of *B. melitensis* vaccine strain Rev1 in a small portion of sheep and goat abortion cases. Despite the recent *B. abortus* burden in ovine, aborted cases were predominantly associated with *B. melitensis* infections in both ovine and caprine, and *B. melitensis* biovar 1 was responsible for the majority of studied cases. These data and the techniques implemented in the present study can shed light on the level of implication of different *Brucella* species in ovine and caprine abortion in Iran. The present study identified *Brucella* agents responsible for abortion in small ruminants at the biovar level. Therefore, it provides precious information for future control programs and vaccination strategies in Middle Eastern regions.

Keywords: Ovine and caprine abortion, *B. melitensis*, *B. abortus*, *B. melitensis* vaccine strain Rev1

Identification des Principales Espèces de *Brucella* Impliquées dans les cas D'avortement Ovin et Caprin par des Méthodes Moléculaires et Classiques

Résumé: La brucellose est reconnue comme un problème majeur de santé publique entraînant des pertes économiques critiques chez les animaux d'élevage. La présente étude a évalué *Brucella spp* isolé de fœtus avortés d'ovins et de caprins (moutons et chèvres) dans différentes régions d'Iran entre 2016 et 2019. Il a utilisé des méthodes classiques et moléculaires afin de déterminer les espèces de *Brucella* présentant des risques plus élevés de complications d'avortement chez ces animaux. Un total de 189 échantillons provenant de 35 cas / séries de cas de lait (16 ovins et 8 caprins), 19 contenus caillés (ovins) et 146 fœtus avortés (116 ovins et 30 caprins) ont été examinés bactériologiquement. Par la suite, les isolats de *Brucella* résultants ont été davantage caractérisés par des approches phénotypiques et moléculaires. La réaction en chaîne par polymérase multiplexe (PCR) (échelle de Bruce) et la PCR basée sur IS711 ont été effectuées sur tout l'ADN extrait pour évaluer la présence de *Brucella spp*. Comme le suggèrent les résultats obtenus, tous les isolats récupérés à partir

d'échantillons d'avortements ovins et caprins étaient soit *B. melitensis*, soit *B. abortus*. L'implication de la souche vaccinale Rev1 *de B. melitensis* dans une petite partie des cas d'avortement ovin et caprin était un sujet de préoccupation. Malgré la récente charge de *B. abortus* chez les ovins, les cas avortés étaient principalement associés à des infections à *B. melitensis* chez les ovins et les caprins, et *B. melitensis* biovar 1 était responsable de la majorité des cas étudiés. Ces données et les techniques mises en œuvre dans la présente étude peuvent éclairer le niveau d'implication de différentes espèces de *Brucella* dans l'avortement ovin et caprin en Iran. La présente étude a identifié les agents *Brucella* responsables de l'avortement chez les petits ruminants au niveau du biovar. Par conséquent, il fournit des informations précieuses pour les futurs programmes de lutte et les stratégies de vaccination dans les régions du Moyen-Orient.

Mots-clés: Avortement ovin et caprin, *B. melitensis*, *B. abortus*, souche *vaccinale B. melitensis* Rev1

Introduction

Brucellosis is a chronic infection mainly caused by *Brucella melitensis* in small ruminants. It has remained an important zoonotic disease in multiple regions across the globe (Castelo and Simões, 2019). Brucellosis is still considered an important endemic zoonosis in numerous emerging and developing countries, as well as some developed regions (Dadar et al., 2019b). In the last two decades, considerable efforts have been made for the eradication and control of brucellosis in caprine and ovine flocks through the culling of seropositive animals and surveillance programs (Blasco and Molina-Flores, 2011).

Nonetheless, due to nomadic and marginal farming systems of ovine and caprine flocks in numerous areas, the control and eradication of Brucella infection remain a difficult task (Blasco and Molina-Flores, 2011). The massive vaccination with the live vaccine of *B. melitensis* strain Rev.1 is recommended when brucellosis is highly prevalent (normally higher than 5%) (Zhang et al., 2018). In Iran, the small ruminant brucellosis is responsible for heavy economic losses, mainly caused by the *B. melitensis* biovar 1 (Behroozikhah et al., 2012; Dadar et al., 2019). Goats and sheep are usually reared in natural lands under pastoralism, grazing cultivation, and extensive system production.

The transmission of *B. melitensis* among ovine and caprine herds may rapidly occur through the introduction of adult males into the flock for breeding, as well as contaminated water and feed. In Iran, brucellosis infects a wide range of animal species with economic importance (Zowghi et al., 2008; Behroozikhah et al., 2012). Up to now, *B. melitensis* biovars 1, 2, and 3 (predominantly 1) have been isolated from buffalo, cattle, goats, dogs, and sheep (Zowghi et al., 2008). Furthermore, *B. abortus* biovars 1, 2, 3, 4, 5, and 9 (predominantly 3) have been reported in cattle, camel, and a small number of sheep (Zowghi et al., 2008; Behroozikhah et al., 2012; Alamian and Dadar, 2019).

Sheep and goats are known as the preferred and classical hosts for *B. melitensis*. The epidemiological, pathological, and clinical features of ovine and caprine brucellosis caused by *B. melitensis* are close to the ones caused by B. abortus infections in cattle. Moreover, small ruminants contribute to the national economy by different products and by-products; moreover, they play an important role in the livelihoods of numerous marginal farmers. In light of the aforementioned issues, the current study aimed to determine the *Brucella* species carrying higher risks of abortion complications in sheep and goats throughout Iran.

Material and Methods

Sample Preparation. The current study examined a total of 189 samples from sheep and goats with a history of abortion, including 24 milk (16 sheep, and 8 goats), 19 abomasum content (sheep), and 146 aborted fetuses (116 sheep, and 30 goats) composed of fetal kidney, liver, abomasum, spleen, heart, and lung. The

samples were sent for analysis to the Department of Brucellosis of the Razi Vaccine and Serum Research Institute (RVSRI, Karaj, Iran) from 2016 to 2019. For *Brucella* culture and isolation, the samples from milk and all visceral organs (kidneys, abomasum content, lungs, and liver) were collected in a sterile plastic bag and preserved at -20°C until analysis. The details of samples are described in Table 1.

Brucella Isolation. For the bacteriological test, all individual milk samples, aborted fetal organs, and abomasum content were subjected to bacterial culture under appropriate protection in safety hoods at the RVSRI Department of Brucellosis. The primary isolation of *Brucella* spp. was performed by inoculating the samples on a *Brucella* selective supplement (containing Bacitracin (12,500 IU), polymyxin B (2,500 IU), Vancomycin (10.0mg) Nystatin (50,000 IU), Cycloheximide (50.0mg), and Nalidixic acid (2.5mg) (Oxoid, UK), and inactivated 5% horse serum in *Brucella* agar (Himedia, India) and incubated for 10 days in 37°C with 10% CO2. The milk samples were centrifuged for 15 min at 3500 RPM, and subsequently, the creamy upper layer and the sediments were cultured. The bacterial cultures were discarded after 10 days of incubation if no growth was visible. The typical colonies of *Brucella* spp. were subjected to further analyses to obtain biotype and full identification.

Biotyping. The biotyping was performed according to Alton et al. (Alton et al., 1988). Monospecific *Brucella* antisera A and M, as well as *Brucella* reference phages, were routinely prepared and applied for analysis and diagnosis of Brucella spp. in our center. A panel of biotyping analysis was performed, including agglutination with specific Brucella antisera, H2S production, CO2 dependence, lysis by specific phages, agglutination by acriflavine, as well as growth in media containing basic fuchsine and thionin. All test results were interpreted according to the OIE manual (http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Brucellosis/).

DNA Extraction and Molecular Typing. Crude genomic DNA of isolated bacteria was extracted by high pure Polymerase chain reaction (PCR) template preparation kit (Ruche, Germany) and stored at -20°C until further analysis. The DNA concentration was analyzed by reading at 260/280 nm using Nanodrop Spectrophotometer (Wilmington, DE, USA). The DNA integrity was then checked by 1% agarose gel. An IS711-based polymerase chain reaction was performed on all extracted DNA to assess the presence of *Brucella* spp. The amplification was performed at a denaturation temperature of 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 55°C for 60 s, 72°C for 3 min, and final extension of 72°C for 10 min (Ewalt and Bricker, 2000).

Species-level molecular determination was also done by multiplex PCR (Bruce-ladder) under the following conditions as 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 56°C for 90 s, 72°C for 3 min, 72°C for10 min (López-Goñi et al., 2008). All PCR reactions were performed in a total volume of 25 µl, by 50 mM KCl, 10 mM Tris–HCl (pH 8), 1.5 mM MgCl2, 0.2 mM each four deoxynucleotide triphosphate and 0.05 IU of Taq polymerase, 0.4 mM of each primer. The amplified products were resolved by electrophoresis using a 1.5 % agarose gel. All the applied primers are described in Table 1.

Results

Brucella isolates (n=57 in total) were obtained from 20 cases out of 35 examined cases/case series (Table 2). These isolates were originated from ovine aborted fetuses (16), ovine milk (1), goat milk (1), or goat's aborted fetuses (2) (Figure 1). The isolated bacteria exhibited typical phenotypic features of *Brucella* spp. All the isolates grew in 10% carbon dioxide (CO_2) after 5 days of incubation at 37°C. The isolated bacteria were gram-negative and formed translucent and shiny small honey-colored colonies with a smooth surface. The isolates were further characterized at the species level, and their identity was confirmed at the biovar

level using abortus, melitensis, ovis, and suis (AMOS)-PCR and Bruce-ladder, respectively. AMOS-PCR experiments identified isolates as B. melitensis, the vaccine strain Rev1 of B. melitensis or B. abortus.

Table 1. Specific primer sets and expected amplicon sizes for different *Brucella* species

Strain Amplicon	Primer set	Primer sequence (5-3')	DNA target	size (bp)	References
B. abortus	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCA T GACGAACGGAATTTTTCCAATCCC	alpha-ketoglutaratedependent dioxygenase	498	(Ewalt and Bricker, 2000)
B.ovis	IS711 *B.ovis*	CGGGTTCTGGCACCATCGTCG	TRAP transporter solute receptor, TAXI family protein	976	(Bricker and Halling, 1995; Ewalt and Bricker, 2000)
B.suis	IS711 B.suis	*GCG CGG TTT TCT GAA GGT TCA GG*	indole-3-glycerol phosphate synthase	285	(Bricker and Halling, 1995; Ewalt and Bricker, 2000)
B. melitensis	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCA T AAATCGCGTCCTTGCTGGTCTGA	hypothetical protein	731	(Bricker and Halling, 1995; Ewalt and Bricker, 2000)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene wboA	1,682	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene bp26	450	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTC GTT CG	Erythritol catabolism, gene eryC (Derythrulose-1-phosphate dehydrogenase)	587	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCA TCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	(López-Goñi et al., 2008)

Table 2. Relative data of animal samples tested for *Brucella* in sheep and goat samples tested for the presence of *Brucella* spp. in different Iranian regions from 2016 to 2019

Case	**Species and biovar isolated**	**Year**	**Province**	Source	Host species	Number of samples	Number of culture-positive samples
1	B.m3	2016	Khorasan Razavi	Aborted fetus	Sheep	1	1
2	B.m1	2016	Mazandarn	Aborted fetus	Sheep	1	1
3	Negative	2016	Fars	Milk	Sheep	1	0
4	Rev1	2016	Kerman	Milk	Goat	8	1
5	Negative	2016	Kerman	Aborted fetus	Goat	1	0
6	Negative	2016	Alborz	Aborted fetus	Sheep	4	0
7	Negative	2017	Yazd	Aborted fetus	Goat	1	0
8	B.m3	2017	Kerman	Milk	Sheep	1	1
9	B.m3	2017	Kerman	Aborted fetus	Sheep	1	1
10	B.m1	2017	Zanjan	Aborted fetus	Sheep	5	4
11	Rev1	2017	Zanjan	Aborted fetus	Sheep	5	1
12	B.m1	2018	Semnan	Aborted fetus	Sheep	1	1
13	Negative	2018	Semnan	Milk Abomasum content	Sheep	6 6	0
14	Negative	2018	Semnan	Aborted fetus Milk Abomasum content	Sheep	6 8 8	0
15	Negative	2018	Fars	Aborted fetus	Sheep	3	0
16	B.m1	2018	Mazandaran	Aborted fetus	Sheep	2	2
17	Negative	2018	Mazandaran	Aborted fetus Abomasum content	Sheep	4 5	0
18	Negative	2018	Fars	Aborted fetus	Goat	5	0
19	Negative	2018	Fars	Aborted fetus	Sheep	16	0
20	B.m2	2018	Alborz	Aborted fetus	Goat	14	7
21	B.m1	2018	Fars	Aborted fetus	Sheep	5	3
22	Negative	2018	Fars	Aborted fetus	Goat	5	0
23	B.m1	2018	Fars	Aborted fetus	Sheep	7	4
24	B.m1	2018	Fars	Aborted fetus	Goat	5	5
24	B.m1	2018	Fars	Aborted fetus	Sheep	7	7
25	B.ab1	2018	Fars	Aborted fetus	Sheep	2	2
26	B.m1	2019	Zanjan	Aborted fetus	Sheep	2	2
27	Negative	2019	Fars	Aborted fetus	Goat	3	0
28	Negative	2019	Fars	Aborted fetus	Sheep	12	0
29	B.m1	2019	Zanjan	Aborted fetus	Sheep	4	4
30	B.m2	2019	Fars	Aborted fetus	Sheep	4	4
31	B.m1	2019	Semnan	Aborted fetus	Sheep	10	4
32	Negative	2019	Alborz	Aborted fetus	Sheep	2	0
33	B.m1	2019	Zanjan	Aborted fetus	Sheep	2	2
34	Negative	2019	Fars	Aborted fetus	Sheep	2	0
35	Negative	2019	Yazd	Aborted fetus	Goat	1	0

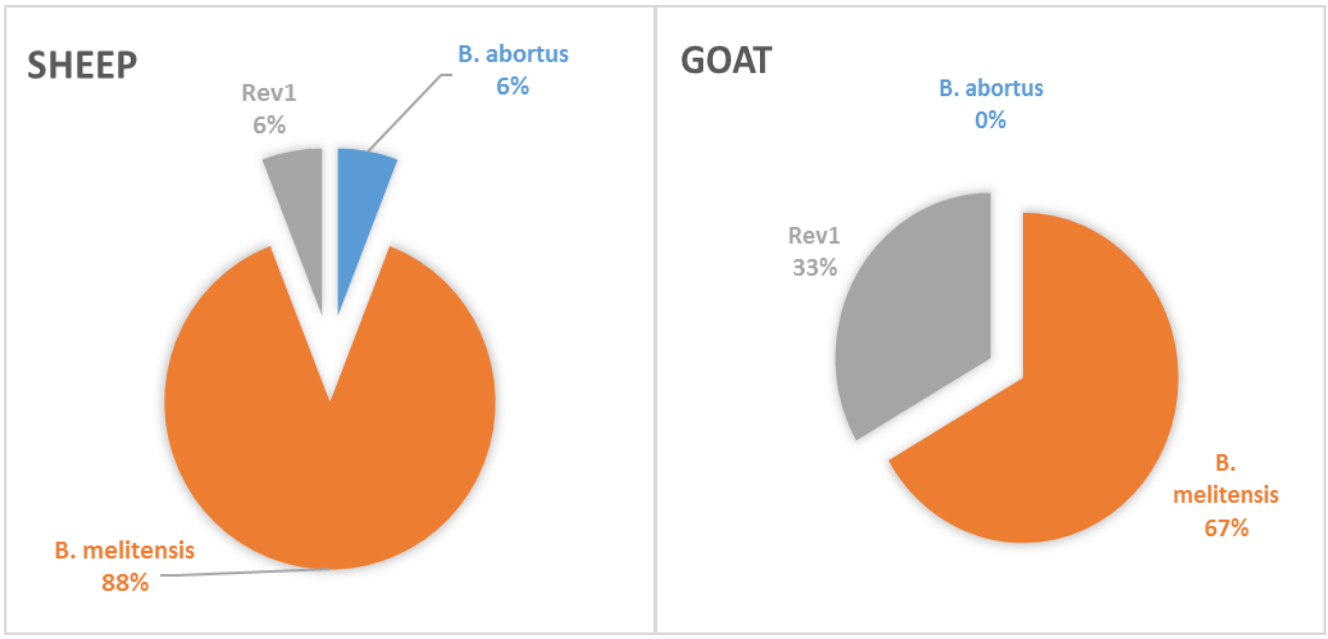

Figure 1. Comparison of *B. melitensis* and *B. abortus* infections in different sheep and goat samples

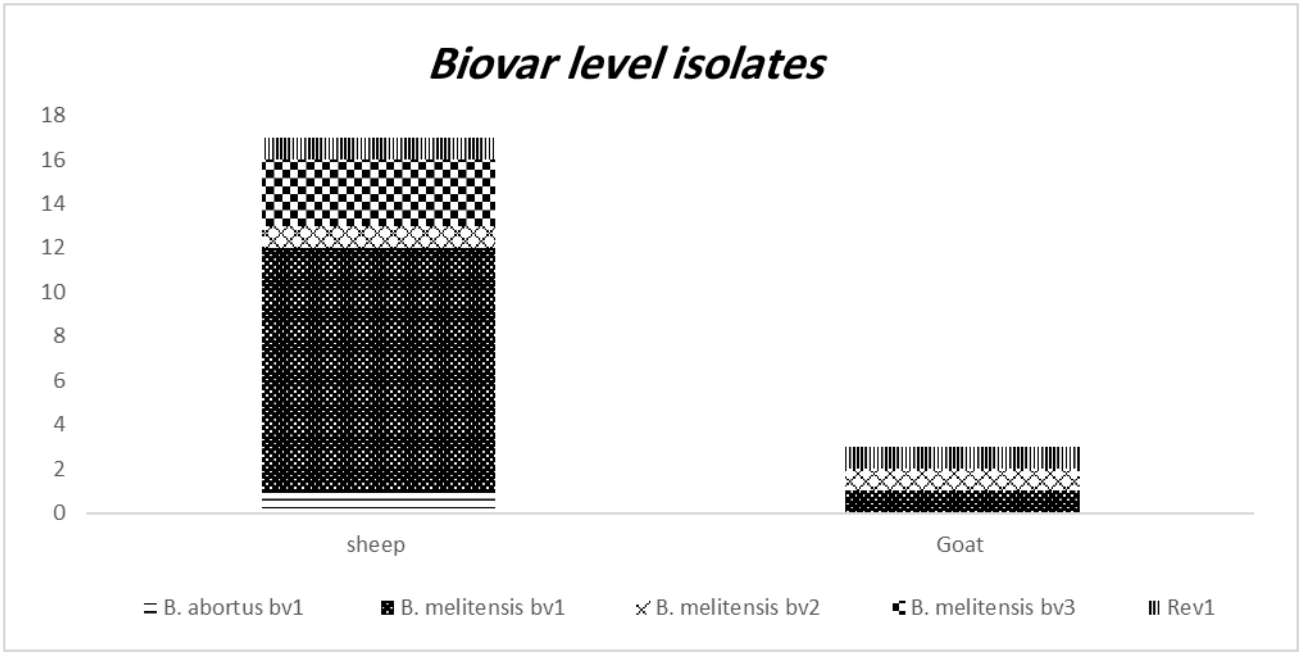

Figure 2. Comparison of *B. melitensis* and *B. abortus* biovars in different sheep and goat samples

Biovar Level Isolates. Consistent with AMOS-PCR results, showing a 498 bp B. abortus specific band, common to biovars 1, 2, and 4 (Bricker and Halling, 1994) (Figure 3), Biotyping confirmed the presence of B. abortus biovars 1 in sheep (1 case). This isolate was confirmed as B. abortus in the Bruceladder PCR as it led to PCR products of 1682, 794, 587, 540, and 152bp in size (Figure 4). A total of 17 B. melitensis strains were isolated from 20 cases /case series, including sheep samples (n=15), as well as few goat samples (n=2), that were identified as wild type B. melitensis and B. melitensis Rev 1 vaccine strain by AMOS-PCR with a product of 731 bp (Figure 3). Although all three B. melitensis biovars were represented, the biovar 1 (n=12) was more common than biovars 2 (n=2) and 3 (n=3) (Figure 2). As evidenced by our Bruce-ladder typing, the final two cases, which were isolated from sheep fetus and goat milk, were identified as B. melitensis Rev 1 vaccine strain. All other isolates were identified as wild type B. melitensis by both AMOS-PCR (PCR product of 731 bp) and Bruce-ladder (PCR products of 1682, 794, 587, 540, 152, and 1,071bp) (Figure 4). The B. melitensis Rev.1 vaccine strain was easily identified from other B. melitensis strains by a specific additional 218-bp fragment (Figure 4).

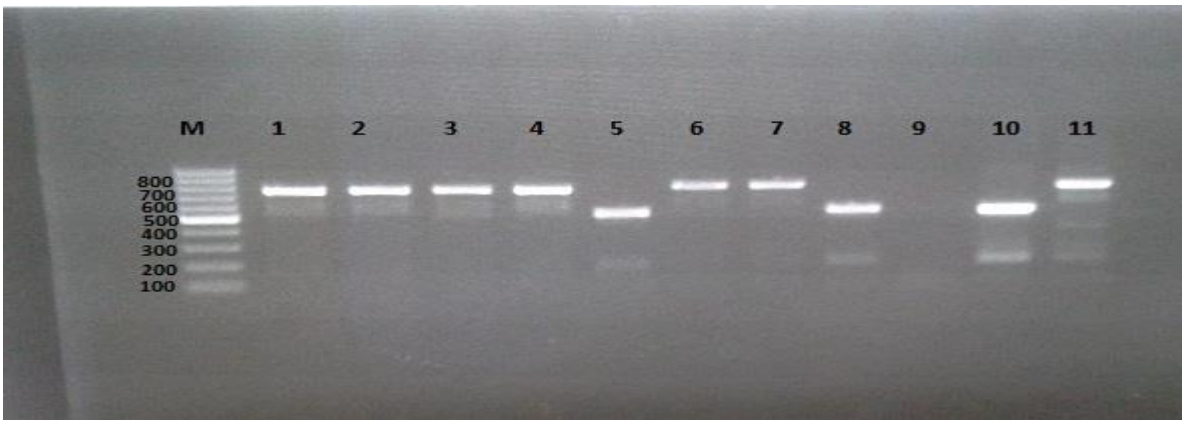

Figure 3. Agarose gel (1%) electrophoresis of PCR amplified products generated from DNA samples in Bruce-ladder PCR. Lane 1 shows a DNA size marker (100bp DNA ladder). Lane 2 demonstrates *B. melitensis* Rev1, Lane 3 and 4: *B. melitensis* field strain, Lane 5: *B. abortus* field strains, Lane 6-7: *B. melitensis* field strain, Lane 8: *B. abortus* field strains, Lane 9: negative control, Lane10: *B. abortus* 544, Lane 11: *B. melitensis* 16M

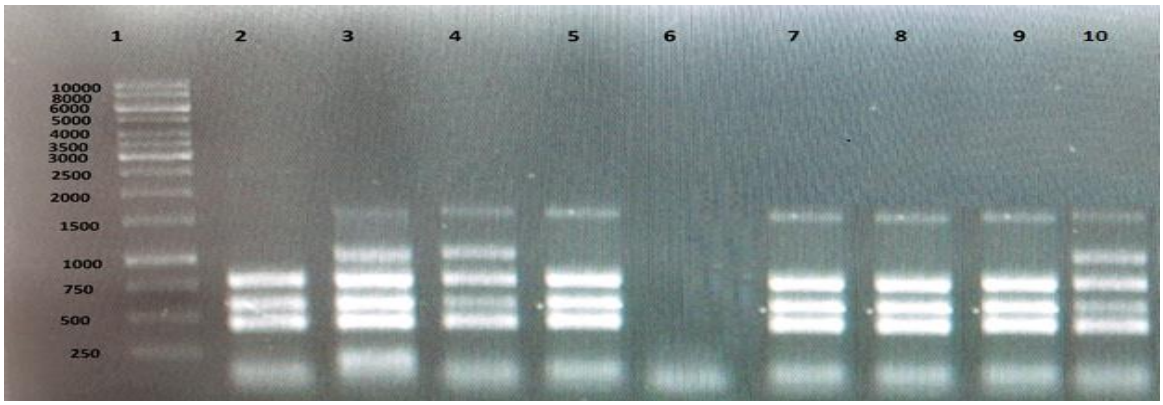

Figure 4. Agarose gel (1%) electrophoresis of PCR amplified products generated from DNA samples in Bruce-ladder PCR. Lane 1 depicts DNA size marker (1000bp DNA ladder), Lane 2 displays *B. abortus* RB51, Lane 3: *B. melitensis* Rev1, Lane 4: *B. melitensis* 16M, Lane 5: *B. abortus* 544, Lane 6: negative control, Lane7-9: *B. abortus* field strains, Lane10: B. melitensis field strain

Discussion

Brucellosis is recognized as one of the major neglected zoonotic diseases with significant economic importance in multiple regions all over the world. Brucellosis in small ruminants is caused by *B.melitensis* and *B. abortus* with some clinical signs including, retained placenta, the birth of weaklings, dead offspring, infertility, and abortion (Castelo and Simões, 2019). It is regarded as an endemic health problem in numerous emerging and developing countries, as well as some developed regions, such as southern and western Europe.

The *Brucella* infection is still widespread throughout most countries of West Asia, some parts of Latin America, and the Mediterranean Basin. Massive vaccination or young female vaccination with the *B. melitensis* strain Rev. 1 is recommended in areas where brucellosis prevalence is high, normally greater than 5% (Blasco and Molina-Flores, 2011). Bacterial culture and isolation are known as the gold standard for the diagnosis of animal brucellosis, followed by biotyping and serological tests.

In multiple Iranian investigations, *Brucella* infection has been assessed by PCR (Dadar et al., 2019) and serology tests (Gharekhani et al., 2016). Nevertheless, there is a dearth of studies exanimating *Brucella* species responsible for caprine and ovine brucellosis at the biovar level (Zowghi et al., 2008; Behroozikhah et al., 2012; Dadar et al., 2019a). Therefore, in the current study, bacteriological and molecular tests were performed (including Bruceladder not previously applied in Iran) to further characterize *Brucella* biodiversity in the ovine and caprine abortion samples.

The present study indicated that passive surveillance for ovine and caprine brucellosis over a four-year period allowed determining the main *Brucella* species and biovars currently responsible for abortion complications in Iran. The results of the current study also pointed to a significant burden of *B. abortus* and *B. melitensis* in sheep. In terms of livestock, while *B. melitensis* infections were common in both sheep and goats (Figure 2), *B. abortus* appeared to be a potential cause of abortion only in sheep. These results are in line with previous observations regarding the isolation of *B. abortus* from sheep, particularly in Nigeria, Brazil, and Egypt (Wareth et al., 2015; Santos et al., 2016).

As suggested by the results of the present study, sheep brucellosis is predominantly related to *B. melitensis* (88%), as well as *B. abortus* (6%) and Rev1 (6%). In a similar vein, caprine brucellosis was also mainly associated with *B. melitensis* (67%), with a much lower burden of Rev1 (33%). These results were in agreement with the view that *B. melitensis* is the most significant caprine pathogen among *Brucella spp*. As depicted in Figure 2, *B. melitensis* biovar 1 was predominantly isolated from sheep, followed by *B. melitensis* biovars 2, *B. melitensis* biovars 3, *B. melitensis* Rev1, and *B. abortus* biovars 1.

Globally, these results are in accordance with the findings of previous studies conducted in different parts of Iran. They have reported that *B. melitensis* biovars 1 is endemic and widely spread in small ruminants (Zowghi et al., 2008; Behroozikhah et al., 2012). Furthermore, *B. abortus* biovar 1 was less prevalent in sheep aborted fetuses (6%). This finding is in agreement with a previous epidemiological study performed in Iran, reporting this biovar as occasional in sheep (Zowghi et al., 2008). According to the results of the present study, *B. melitensis* biovars 1, *B. melitensis* biovars 2, *B. melitensis* biovars 3, *B. melitensis* Rev1, and *B. abortus* biovars 1 are the only species that have been isolated in sheep and goat fetus abortion cases.

B. melitensis biovar 1 was first reported in case of a sheep in central Iran (Isfahan) and then spread to different Iranian regions, infecting sheep and goats, as well as cattle, camels, dogs, and humans. The study performed by Ashrafganjooyi et al. on 700 samples reported *B. melitensis* biovar 1 as the most common biovar in sheep and goat milk samples (Ashrafganjooyi et al., 2017). *B. melitensis* biovar 1 was also reported in Israel (Banai et al., 1990), Libya (Gameel et al., 1993), Oman (Refai, 2002), China (Jiang et al., 2011), Iran (Zowghi et al., 2008; Behroozikhah et al., 2012), and

Kenya (Muendo et al., 2012). Moreover, *B. abortus* biovar 1 was reported in Nigeria (Ocholi et al., 2005), Egypt (Wareth et al., 2015), Brazil (Santos et al., 2016), and Iran (Zowghi et al., 2008).

The results of the present study demonstrated that *B. melitensis* biovar 1 is the most common cause of ovine and caprine abortion in Iran. The *B.melitensis* biovar 2 was only reported in sheep and goats from Fars and Alborz provinces. Despite being the most prevalent biovar in China (Jiang et al., 2011), it has been less frequently reported in Mediterranean and Middle East countries. *B. melitensis biovar 2* was previously reported in Iran, Turkey, and Saudi Arabia (Refai, 2002; Behroozikhah et al., 2012; Dadar et al., 2019a), and the obtained data pointed to the incidence of this type in aborted sheep and goat of two provinces.

In the current study, *B. abortus* biovar 1 was isolated from a single sheep sample. The isolations of *B. melitensis* Rev1 from caprine milk and ovine fetus revealed the potential shedding of Rev1 in milk, as well as its capacity, to cause abortion in small ruminants, especially if the vaccination timing is not optimal. The live *B. melitensis* Rev 1 strain is currently applied as the exclusive vaccine for the prevention of goat and sheep brucellosis in Iran (Banai, 2002). The full- and reduced-doses of Rev 1 have been both suggested as safe and effective approaches for controlling small ruminant brucellosis.

All things considered, the obtained findings, utilizing both classical and newly introduced molecular approaches, indicated that various *Brucella* strains are responsible for ovine and caprine abortion in Iran. Moreover, it was found that *B. melitensis* and *B. abortus* biovars are implicated to a certain extent. Although *B. melitensis* biovar 1 is the most virulent sheep and goat pathogen among the genus, other *B. melitensis* biovars, as well as *B. abortus* biovar 1 (in sheep), could potentially cause abortion in infected cases. Therefore, the approach used in the current study, based on both bacterial culture and PCR methods, can provide critical data on the prevention and control of the disease, as well as the selection of appropriate vaccination strategies.

Authors' Contribution

Study concept and design: M. D.
Acquisition of data: S. A.
Analysis and interpretation of data: M. D.
Drafting of the manuscript: S. A.
Critical revision of the manuscript for important intellectual content: S. A.
Statistical analysis: M. D.
Administrative, technical, and material support: S. A.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the authorship and/or publication of this article.

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